BRIEF COMMUNICATIONS

Building an Atlas of Subcellular Localization Markers in WEHI-231 Cells

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Abstract: A goal of the Alliance for Cellular Signaling (AfCS) Microscopy Laboratory is to generate a visual library of subcellular structures that can be used as a reference guide for localization studies. Our model system for initial characterization of these markers is the WEHI-231 cell line. We have collected a variety of fluorescently tagged protein markers that localize to specific intracellular compartments, including cytosol, nucleus, nuclear membrane, endoplasmic reticulum, Golgi, plasma membrane, and cytoskeletal structures. This presentation briefly reviews our current collection of subcellular markers and offers examples of their localization pattern and temporal dynamics.

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Introduction

An important approach to understanding the complexities of signal transduction is studying the spatial organization of signaling molecules among subcellular compartments. Knowledge of the localization patterns and behaviors of these molecules can help define the regulatory mechanisms utilized by a cell to control transmission of intracellular signals. Our strategy for gathering information about subcellular localization is, first, to express fluorescently tagged signaling molecules of interest and make a subjective determination of their location using confocal microscopy. Second, by choosing subsets of subcellular markers that are also fluorescently tagged to coexpress with the signaling molecule, we can then define specific localization patterns based on observed colocalization. We will continue to expand our list of localization markers as we encounter novel localization patterns. We present this brief overview as an initial foundation for our atlas of subcellular markers.

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Molecular biology: Specific molecules were chosen as markers primarily from searching the literature for previously identified organelle markers. We used localization domains instead of full-length proteins in cases where there is evidence that those protein regions are sufficient to target a fluorescent tag to the location of interest. When a full-length coding sequence was required, we used mouse sequences. Unless otherwise specified, a single fluorescent protein was used as the fluorophore, and in this report, only yellow fluorescent protein (YFP)-tagged markers were used. In two instances, a 6-tandem YFP tag was used. Many of the markers described here are available from Clontech. In the near future, we plan to make noncommercial marker constructs available to the academic community and to provide further details of how each was generated.

Cell culture and transfection: WEHI-231 cells were cultured in supplemented RPMI medium 1640, as described in detail in the AfCS protocol PP00000111. Cells were transfected with plasmid DNA by electroporation 16 to 24 hours prior to visualization (PP00000113). For microscopy, cells were plated onto 8-well coverglass chambers (PP00000114).

Imaging: Confocal images of cells were acquired with a cooled charge-coupled-device (CCD) camera (Roper Scientific Photometrics CoolSNAP HQ) and viewed using a Yokagawa spinning disk confocal head from PerkinElmer attached to the side port of a Zeiss Axiovert 200 M microscope. Details of image acquisition and processing are available in the AfCS protocol PP00000135.

Subcellular Markers

The following pages list and describe sample subcellular markers that are currently used in the AfCS Microscopy Laboratory. Table 1 lists the identity, specificity, and construct name for each marker. Images of WEHI-231 cells expressing each of these markers follow. Images represent three planes along the z-axis that were acquired to sample the bottom, middle, and top of the cell fluorescence. Bright field images were acquired at the same planes as the static fluorescent images. Time-lapse images (30; 1 to 5 seconds apart) at one plane were acquired prior to the static images. The range of fluorescent intensities that is represented on an image is shown on the scale bar at the bottom left of each panel. The intensity values vary along each plane and should be noted when comparing images. Three examples of the utility of these markers are shown on pages 18-20.

Table 1. ***Sample markers for subcellular localization studies.***

Marker	**Subcellular region**	**Construct name**
Single fluorescent protein	Cytosol and nucleus	pEYFP-N1
Tandem fluorescent proteins (YFP6)	Cytosol	A97GYFP6CXTK
NLS of SV40 large T antigen x 3 (Nuc)	Nucleus (with heavily stained nucleolus)	pEYFP-Nuc
YFP6-NLS	Nucleus	A97XM003B2TK
Lamin B receptor	Nuclear membrane	A05XM011B1NK
Calreticulin+KDEL	Endoplasmic reticulum	pEFYP-ER
Golgin	Golgi	A08XM005B1TK
Myristylation and palmitylation sequence of neuromodulin (Mem)	Plasma membrane and perinuclear region	pEYFP-Mem
Farnesylation and polybasic motif of K-Ras4b	Plasma membrane	A08XM036B1TK
α-Tubulin	Microtubules	A08XM020A1TK
γ-Tubulin	Centrosomes	A05XM021A1NK
Arp3	Polymerizing actin	A08XM035A1TK

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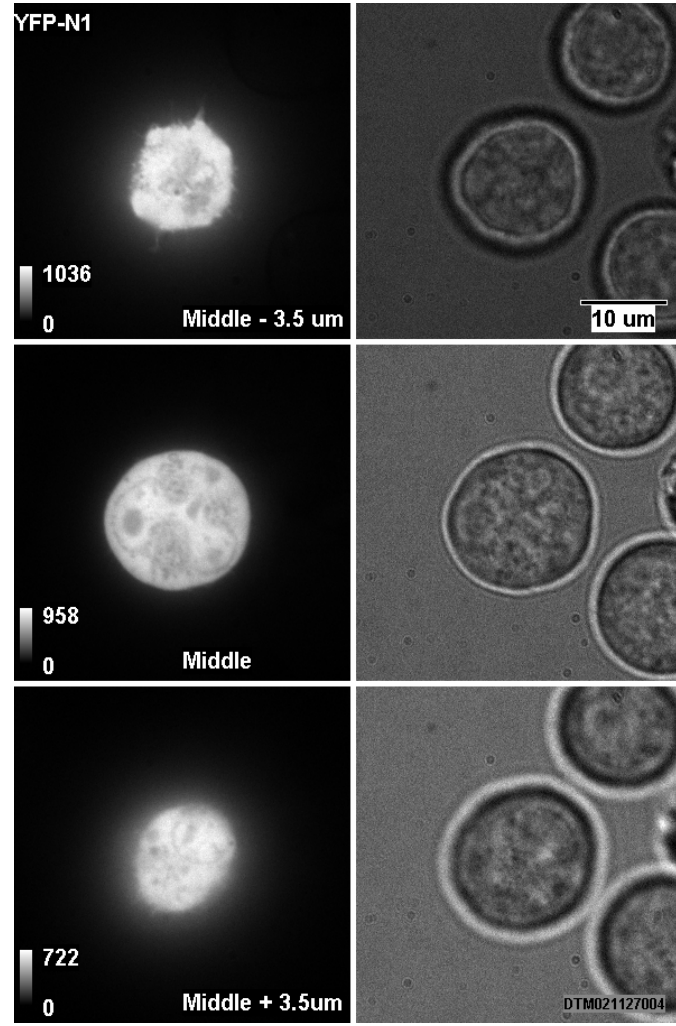
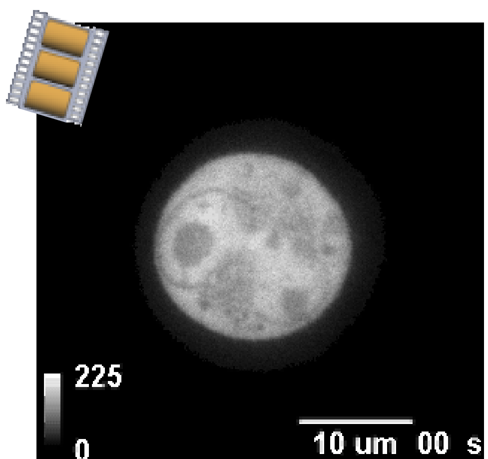
Cytosol and Nucleus

Fluorescent protein tags (alone) typically distribute themselves throughout the cytosol. This example shows that YFP (Clontech, cat. no. 6006-1) is only excluded from some vesicular and membranous organelles that appear as a darker "counterstain." This marker is helpful for the colocalization of signaling molecules that distribute freely throughout the cell.





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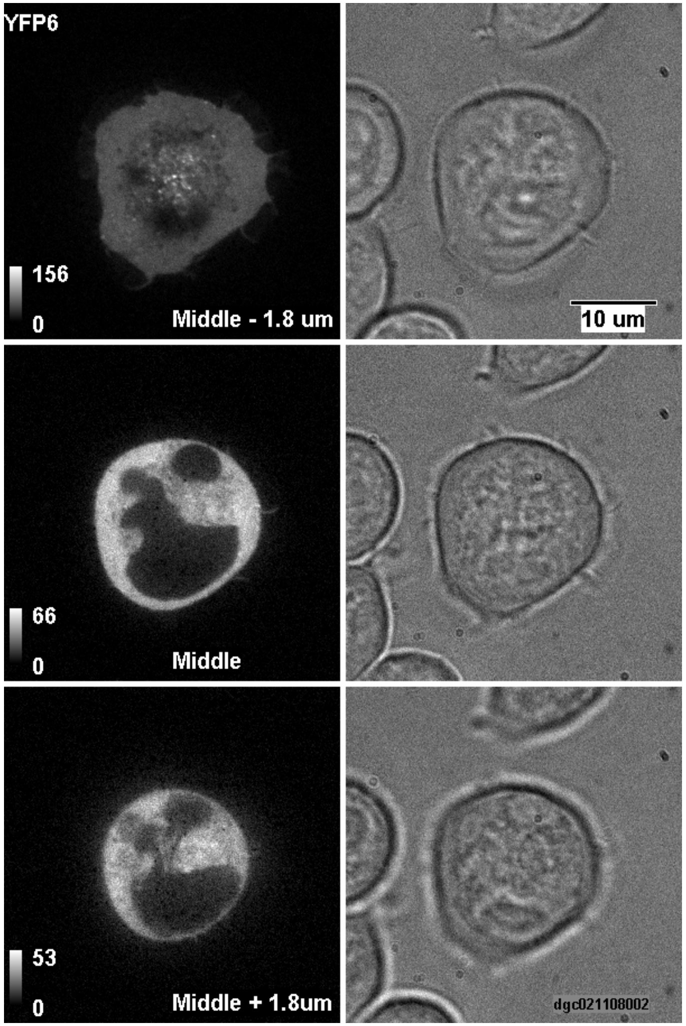
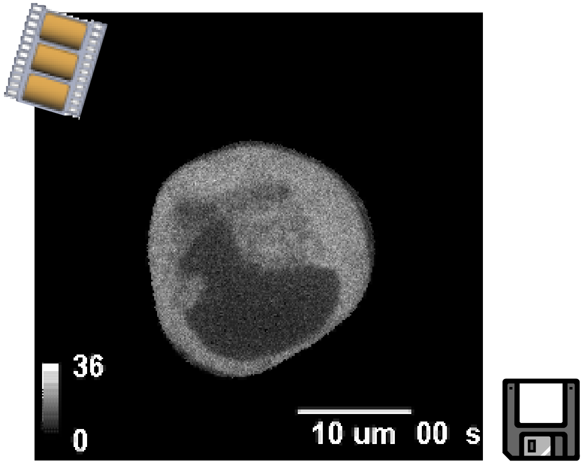
Cytosol

To create a marker that is excluded from the nucleus, but is otherwise widely distributed, we generated a construct with six tandem repeats of YFP. This protein is predicted to be close to 180 kD and is not expected to passively diffuse into the nucleus.





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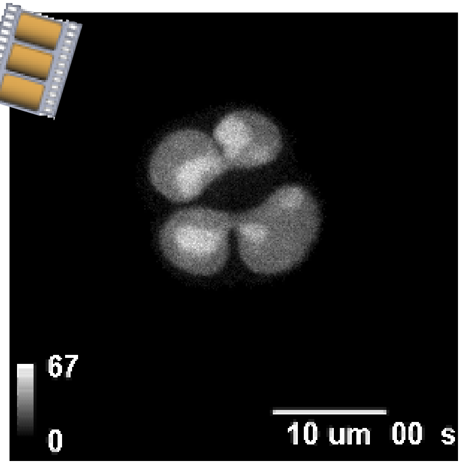
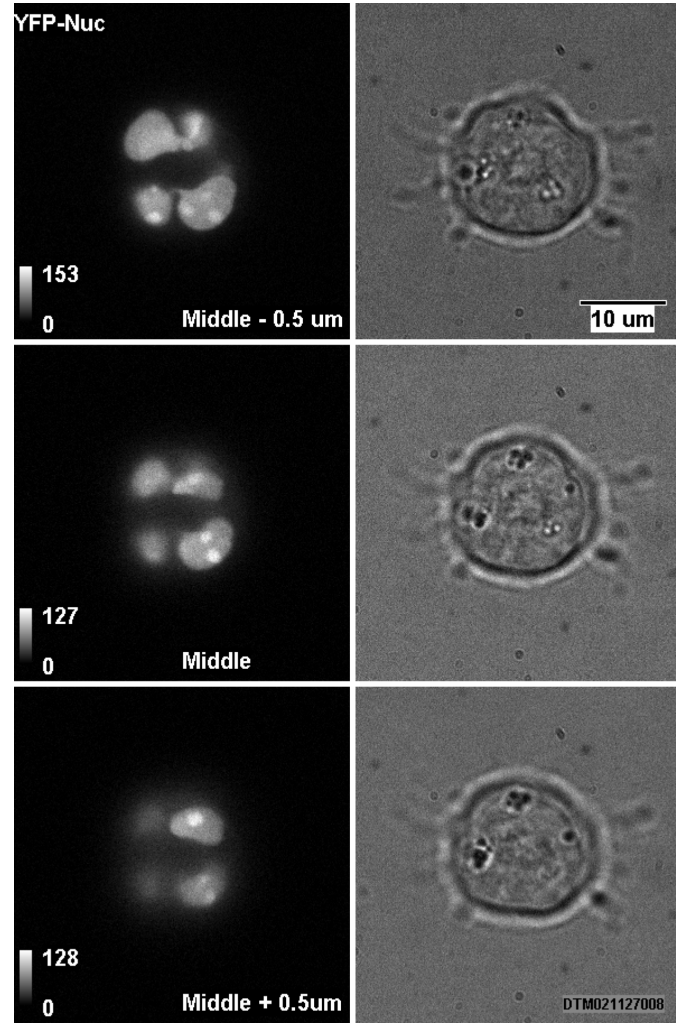
Nucleus (with heavily stained nucleolus)

A construct with three tandem repeats of the nuclear localization signal of simian virus large T antigen joined to the C-terminus of YFP produces a marker that specifically targets the nucleus and demonstrates enrichment in the nucleolus. The construct encoding this chimera is available from Clontech (cat. no. 6905-1).





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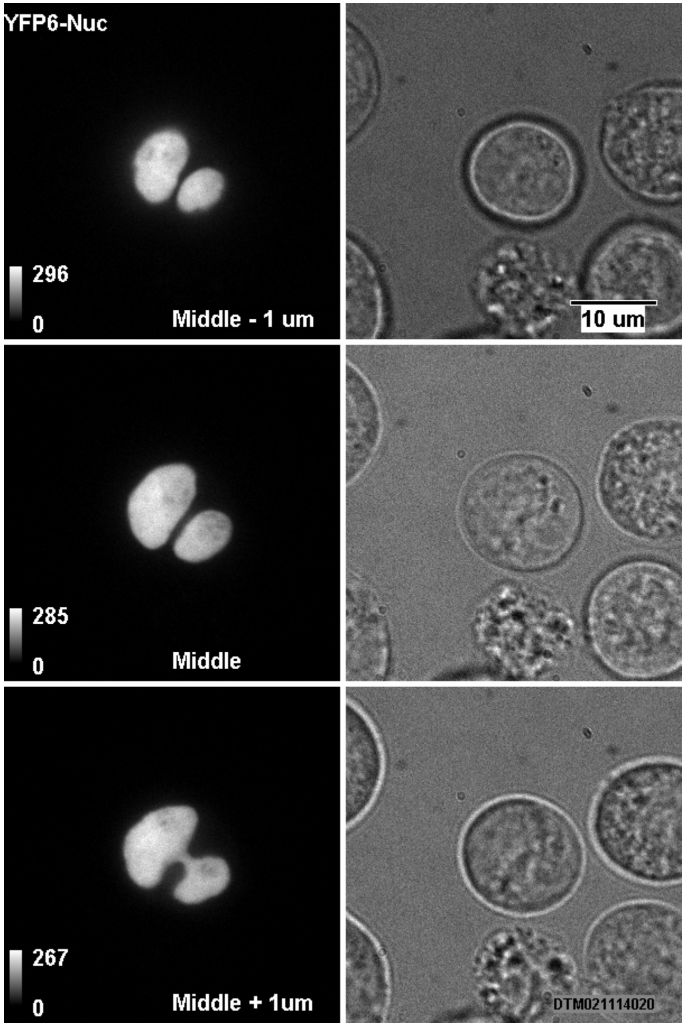
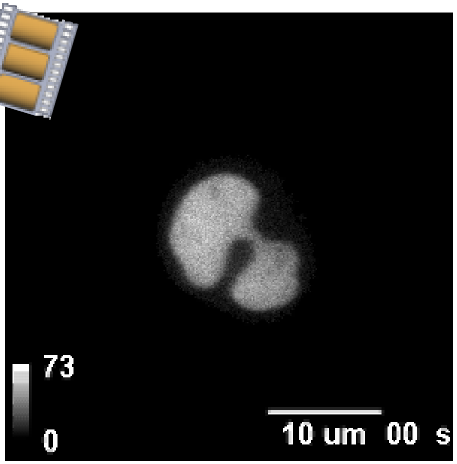
Nucleus (even distribution of fluorescent protein)

Three tandem repeats of the nuclear localization signal of simian virus large T antigen were joined to the C-terminus of six tandem YFP molecules. This protein targets to the nucleus but is not enriched in the nucleolus.





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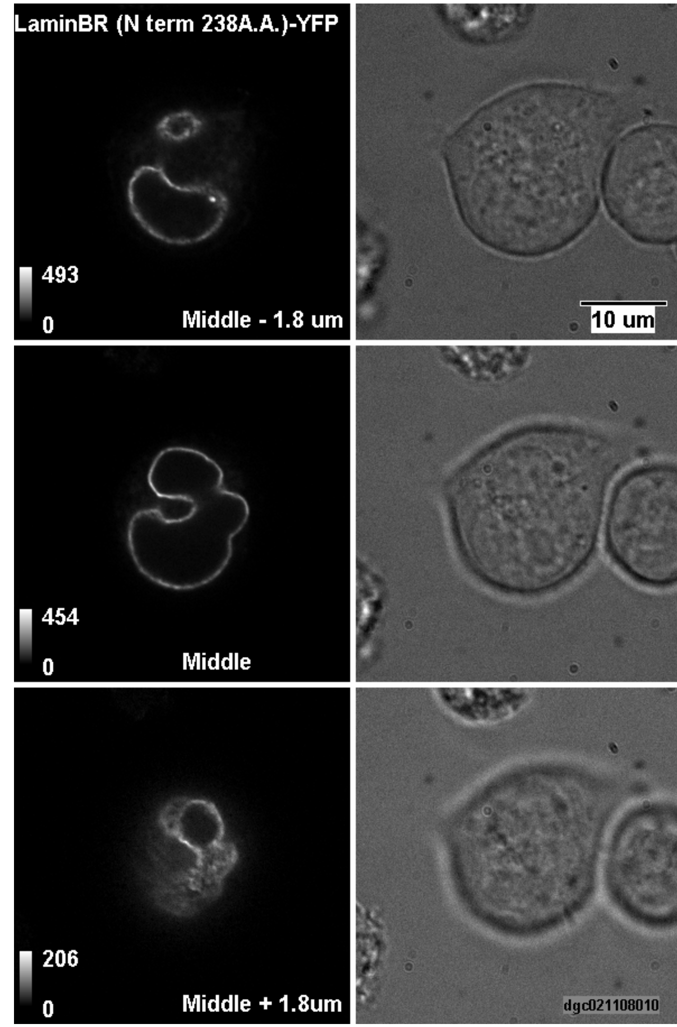
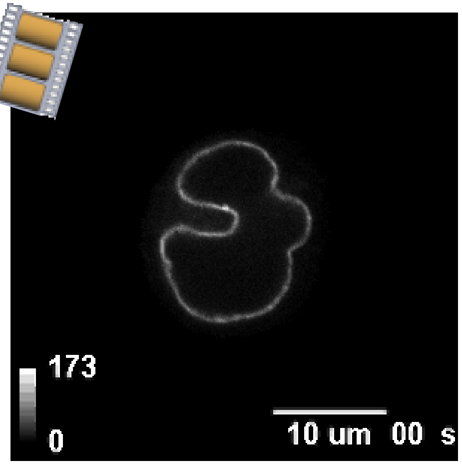
Nuclear Membrane

The nuclear envelope is a dual membrane contiguous with the the endoplasmic reticulum. To identify the nuclear membrane, a fluorescent protein was placed on the C-terminal end of the first 238 amino acids of the mouse lamin B receptor. The receptor sequence consists of the nucleoplasmic tail and the first transmembrane domain; thus, the fluorescent tag is placed in the lumen of the endoplasmic reticulum (1).





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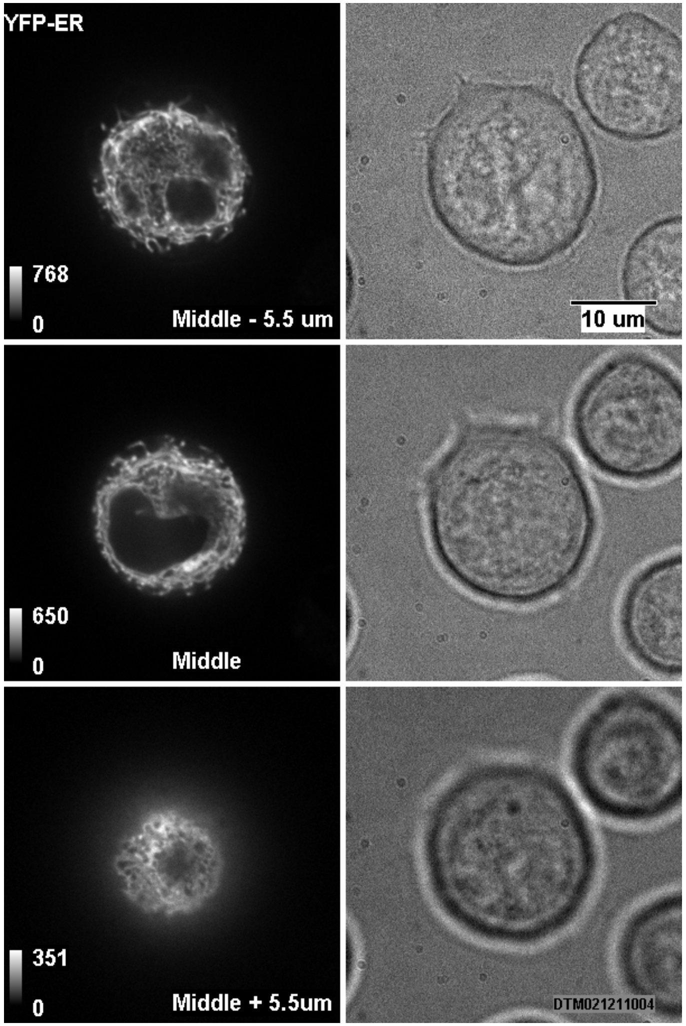
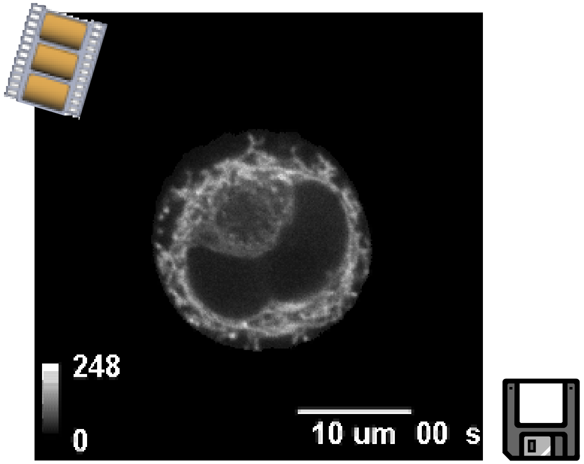
Endoplasmic Reticulum

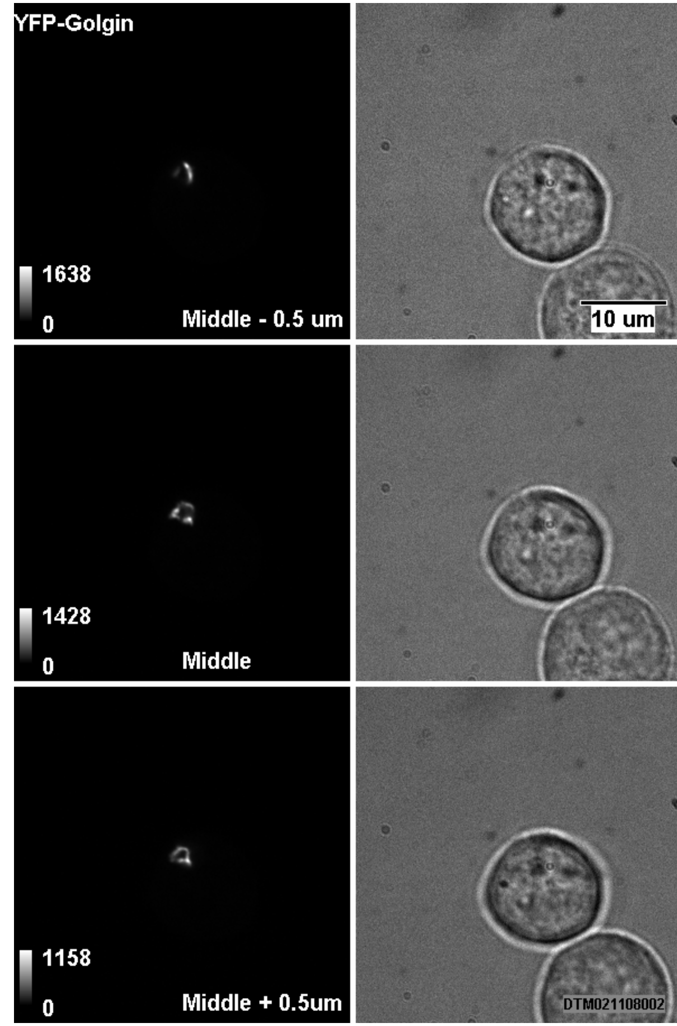
To identify the endoplasmic reticulum (ER), we utilized a marker from Clontech (cat. no. 6906-1). This construct encodes the fluorescent tag flanked by the ER targeting sequence of calreticulin at the N-terminus and the sequence for the ER retrieval sequence, KDEL, at the C-terminus.

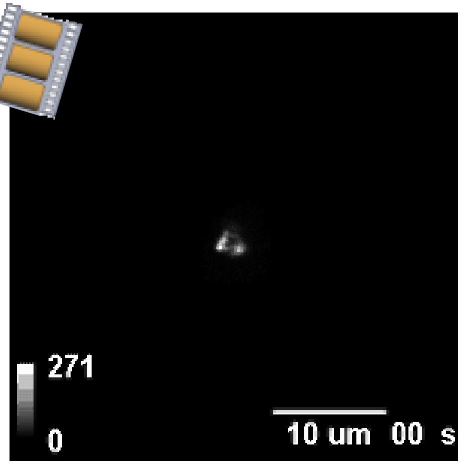




Golgi

This construct encodes a fusion protein consisting of the C-terminal 72 amino acids of Golgin-245 and the fluorescent protein. This region contains a GRIP domain that has been reported to bind the cytoplasmic surface of Golgi membranes (2,3).





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Plasma Membrane (nonspecific)

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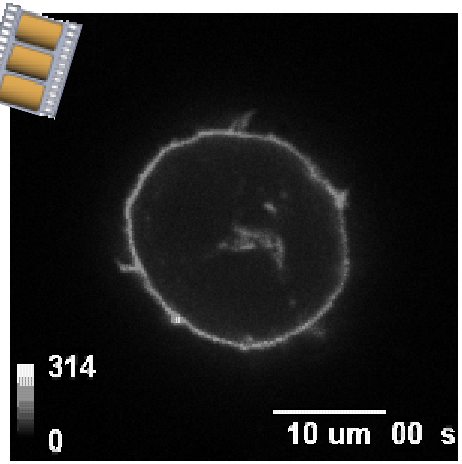
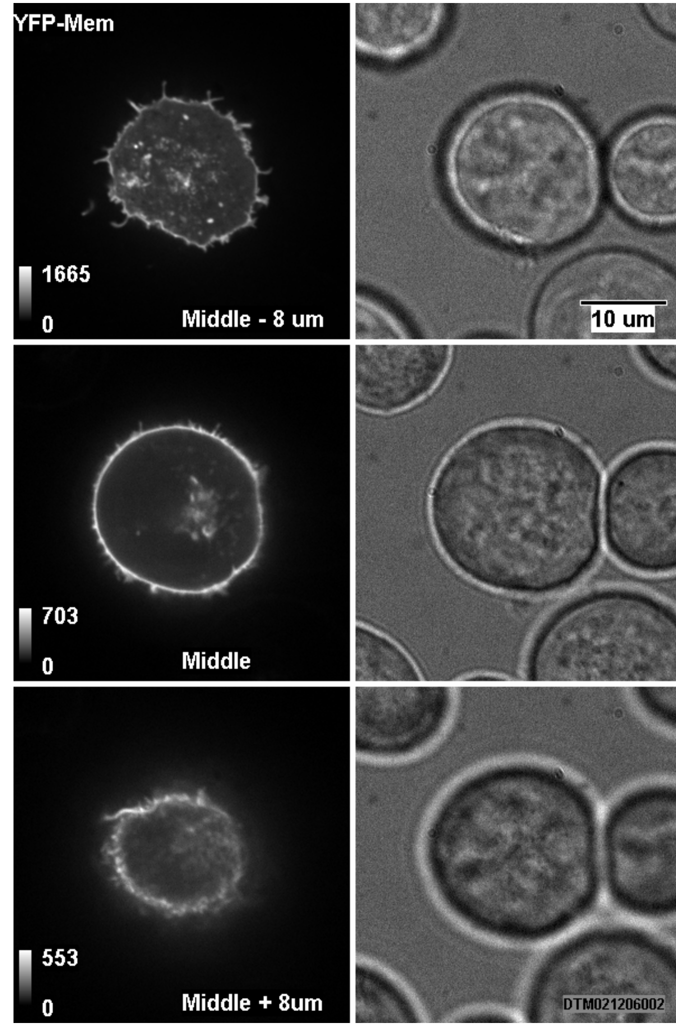
To locate the plasma membrane, we used a construct from Clontech (cat. no. 6917-1) that encodes a fusion protein consisting of the N-terminal 20 amino acids of neuromodulin (GAP-43) and YFP. The 20-amino-acid fragment contains a signal for posttranslational palmitoylation of cysteines 3 and 4. This modification targets proteins to membranes but not specifically to the plasma membrane, as is evident by the intracellular staining in these images.





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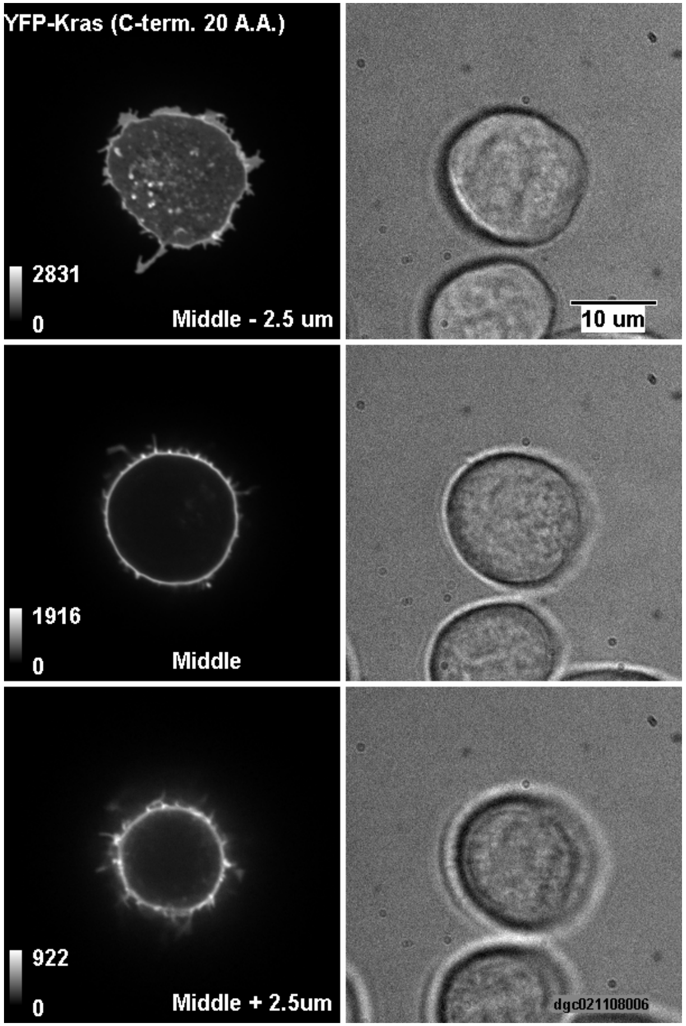
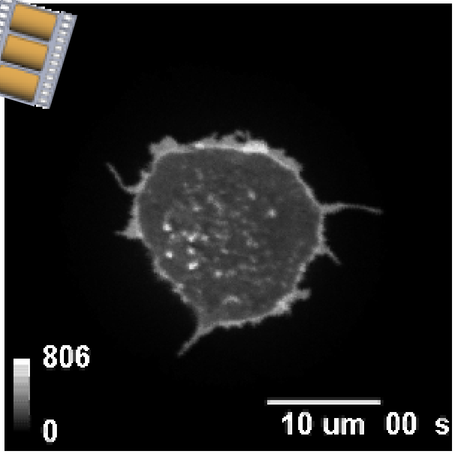
Plasma Membrane (specific)

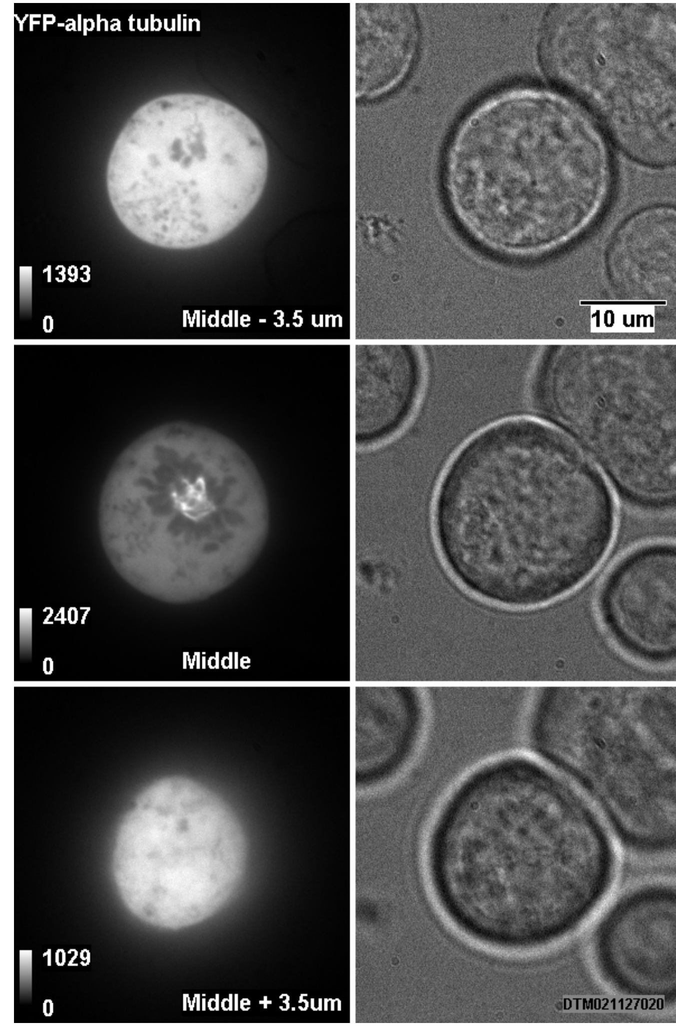
A fusion protein of YFP and the C-terminal 20 amino acids of human K-Ras can achieve increased plasma membrane specificity. The 20-amino-acid fragment contains a signal for posttranslational farnesylation and a polybasic motif (4). The diffuse cellular fluorescence seen in the upper panel is from the plasma membrane at the glass interface; thus, the marker specificity is best appreciated in the middle plane. The movie (below) was captured near the glass surface to show the membrane dynamics of this area.

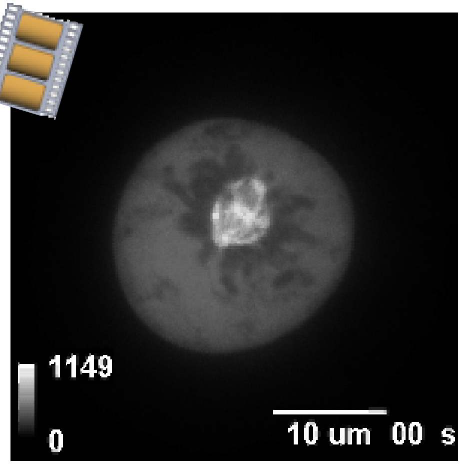




Microtubules

Microtubules are identified using a fusion protein consisting of YFP and full-length mouse α-tubulin. A similar construct encoding human α-tubulin is available from Clontech (cat. no.6118-1). Note that the cytosol has similar intensity values in all planes, but the much brighter tubular structures seen in the middle panel increase the image's intensity range and make the cytosol appear dimmer in that plane.





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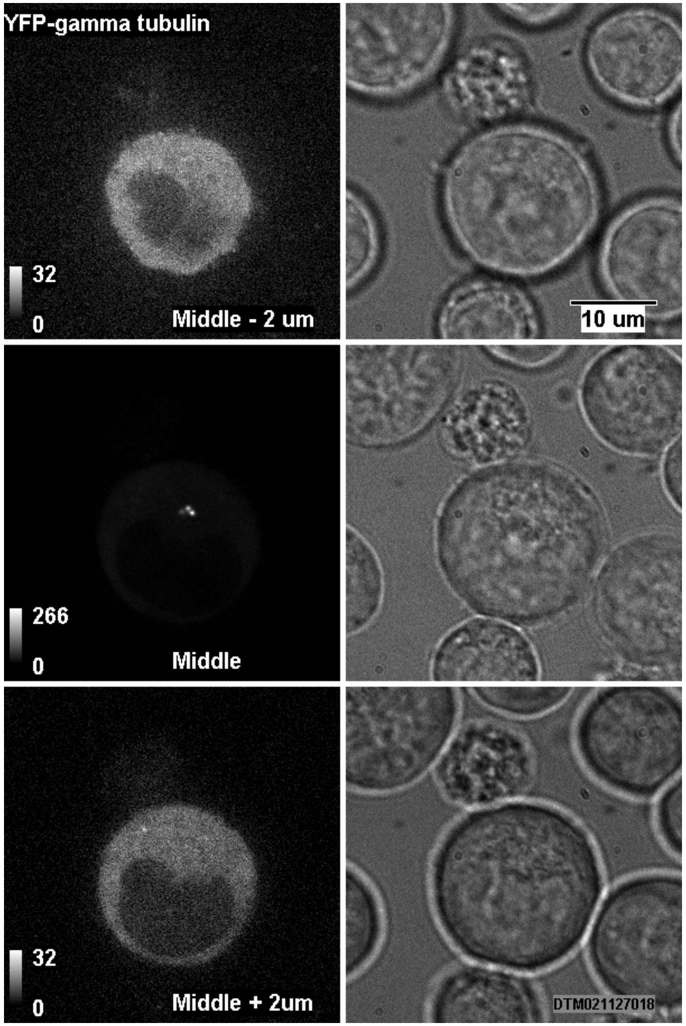
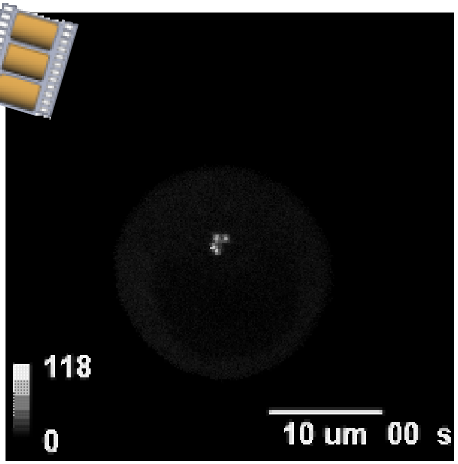
Centrosomes

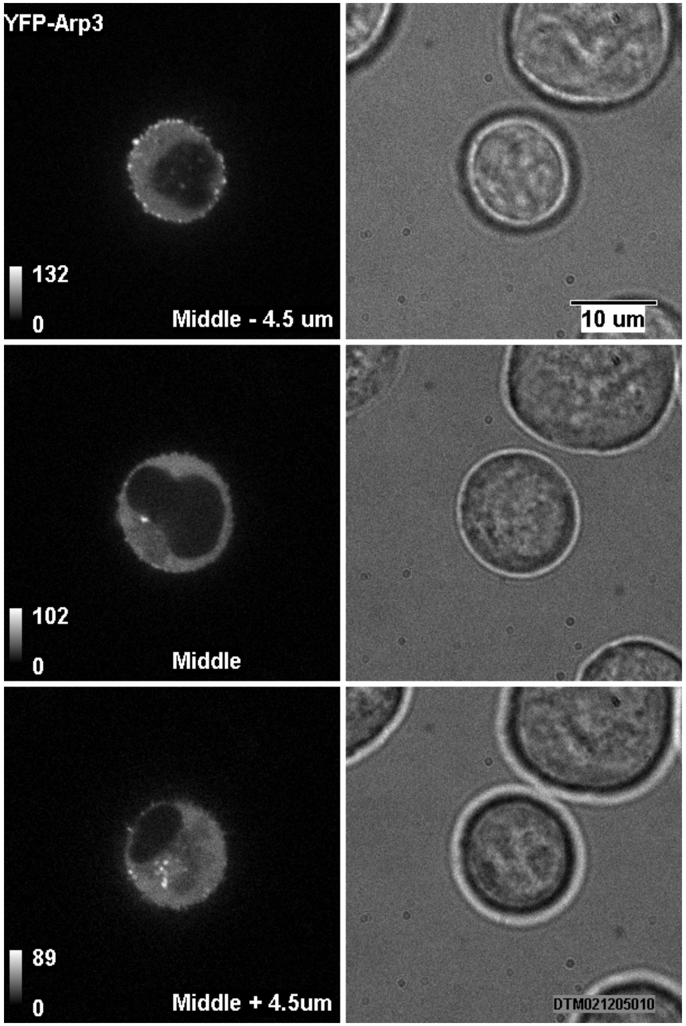
Centrosomes can be identified with a fusion protein consisting of full-length mouse γ-tubulin and YFP. In many cells the marker appears to be purely cytosolic, but in some cells one can clearly see one or two bright punctate structures in one z position. Note that the cytosol has similar intensity in all planes, but the much brighter structures seen in the middle panel increase the image's intensity range and make the cytosol appear dimmer in that plane.

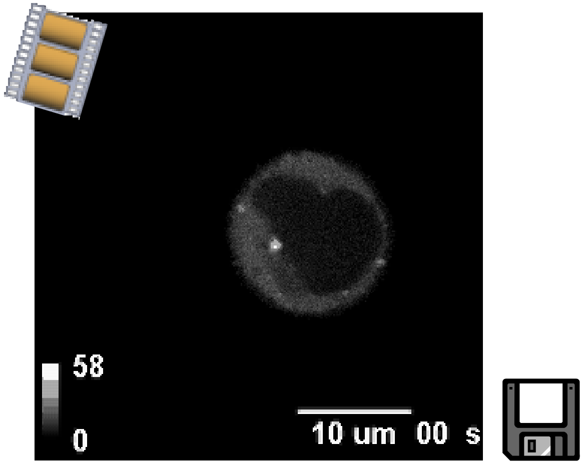




Polymerizing Actin

This construct encodes a fusion protein of YFP and the actin-related protein Arp3. The Arp 2/3 complex concentrates at active sites of actin polymerization (5).





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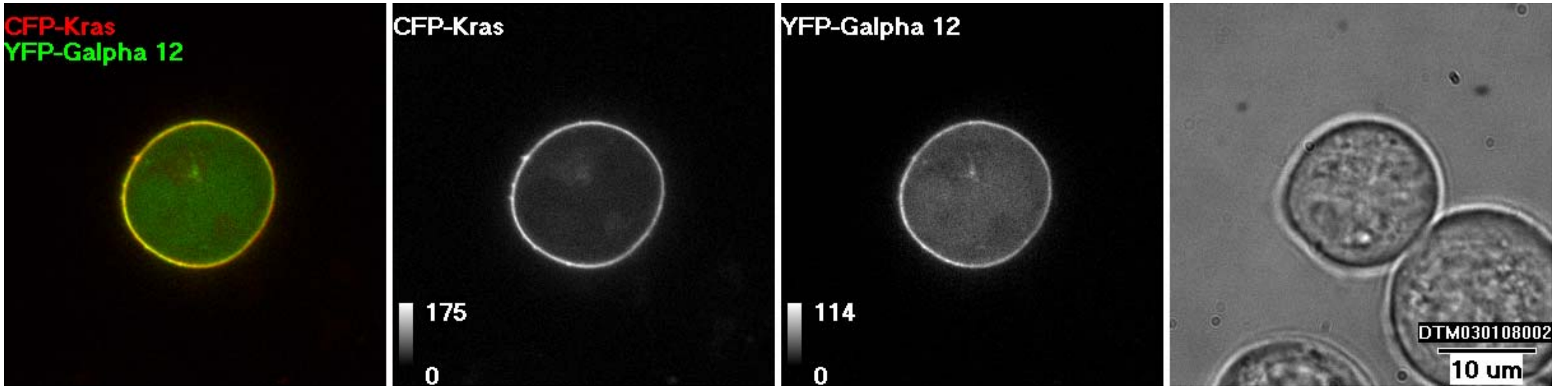
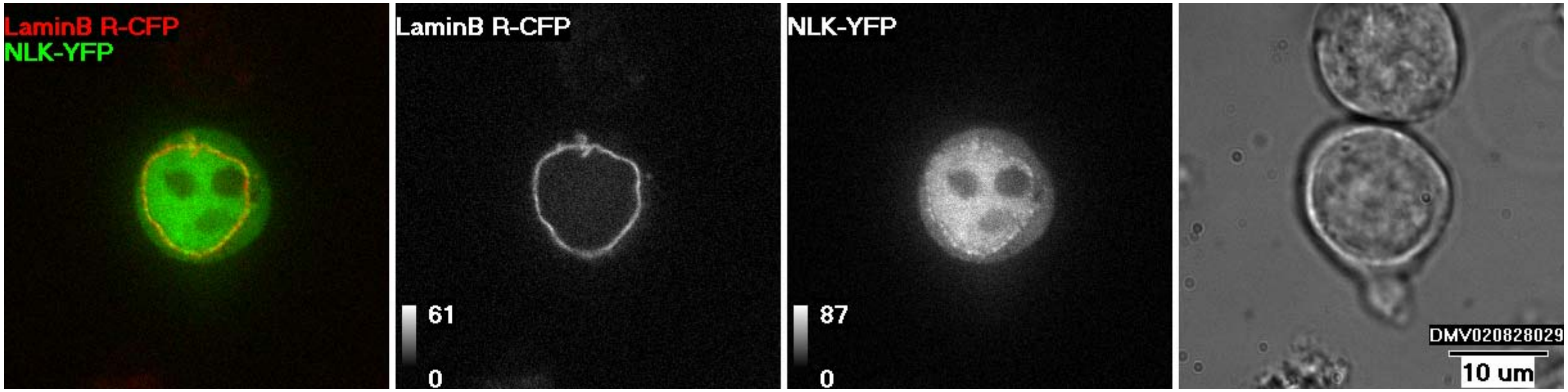


Above left is a two-color overlay of the plasma membrane localization sequence of K-ras, with an N-terminal cyan fluorescent protein (CFP) tag, and G protein alpha 12 (Galpha 12; AfCS PID A000039), with an N-terminal YFP tag. The marker colocalizes with the fraction of G protein that is expressed in the plasma membrane, but there is a less prominent cytosolic and nuclear fraction as well.

AfCS barcode for the YFP-G alpha 12 plasmid: A08XP025A1TK

Utility of the Markers



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Above left is a two-color overlay of lamin B receptor tagged with a C-terminal CFP tag and a nemo-like serine-threonine kinase (NLK, AfCS PID A001668) with a C-terminal YFP tag. In some cells, NLK is found in a punctate pattern at the nuclear membrane. The lamin B receptor identifies the nuclear membrane but does not fully "describe" the localization pattern of NLK.

AfCS barcode for the NLK-YFP plasmid: A05XK050A1NK

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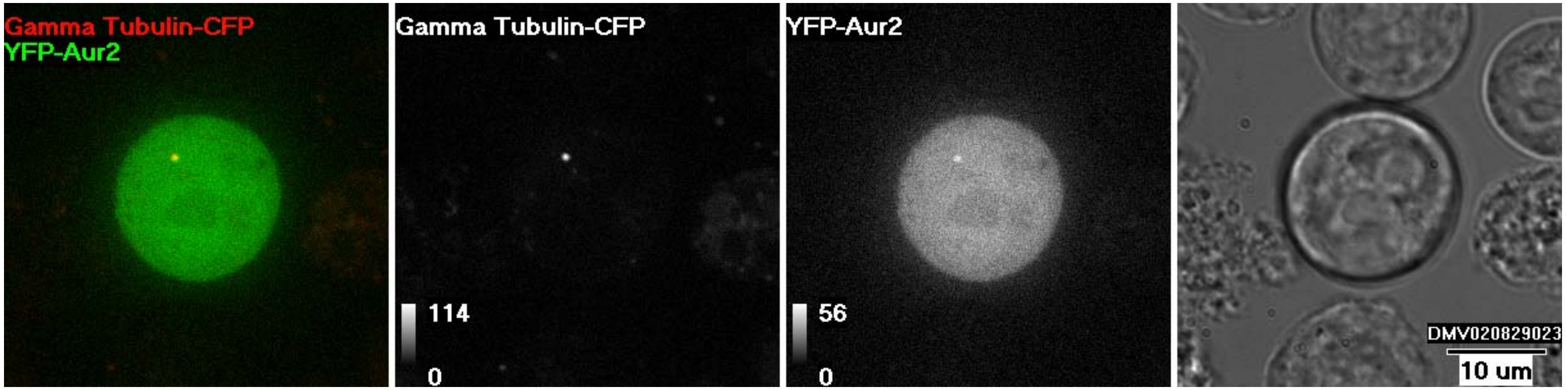

Above left is a two-color overlay of a cotransfection of γ-tubulin with a C-terminal CFP tag and an Aurora 2 serine-threonine kinase (AfCS PID A000352) with an N-terminal YFP tag. In some cells, the kinase is found at one spot that localizes with γ-tubulin. This is consistent with previous reports of Aurora-related kinases localizing to centrosomes (6).

AfCS barcode for the Aurora 2-YFP plasmid: A08XK104A1TK

Table 2. ***Additional subcellular markers.***

Marker	**Subcellular region**
ERGIC-53	ER-Golgi intermediate compartment
Cytochrome C-oxidase subunit 8a	Mitochondria
Galactosyltrasferase	Golgi
Vimentin	Intermediate filaments
VAMP 3/Cellubrevin	Recycling endosomes
Tandem FYVE domain of HRS	Sorting endosomes
Vamp1a, 2, 4, 5, 7, 8, mammalian sec22, mammalian YKT6	Other markers of the exocytic and endocytic pathways

This table lists additional subcellular markers that have been or will be tested in the laboratory, but for which images are not shown.

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